

## Mitochondrion as a Novel Target of Anticancer Chemotherapy

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Mitochondrial membrane permeabilization is a critical event in the process leading to physiologic or chemotherapy-induced apoptosis (programmed cell death). This permeabilization event is, at least in part, under the control of the permeability transition pore complex (PTPC). Oncoproteins from the Bcl-2 family and tumor suppressor proteins from the Bax family interact with PTPC to inhibit or facilitate membrane permeabilization, respectively. Conventional chemotherapeutic agents elicit mitochondrial permeabilization in an indirect fashion by induction of endogenous effectors that are involved in the physiologic control of apoptosis. However, an increasing number of experimental anticancer drugs, including lonidamine, arsenite, betulinic acid, CD437, and several amphipathic cationic  $\alpha$ -helical peptides, act directly on mitochondrial membranes and/or on the PTPC. Such agents may induce apoptosis in circumstances in which conventional drugs fail to act because endogenous apoptosis induction pathways, such as those involving p53, death receptors, or apical caspase activation, are disrupted. However, stabilization of the mitochondrial membrane by anti-apoptotic Bcl-2-like proteins reduces the cytotoxic potential of most of these drugs. Targeting of specific PTPC components may overcome this Bcl-2-mediated apoptosis inhibition. One strategy involves cross-linking of critical redox-sensitive thiol groups within the PTPC; another involves the use of ligands to the mitochondrial benzodiazepine receptor. Thus, the design of mitochondrion-targeted cytotoxic drugs may constitute a novel strategy for overcoming apoptosis resistance. [J Natl Cancer Inst 2000;92:1042-53]

It is well established that apoptosis (programmed cell death) plays a pivotal role in tissue homeostasis and that inhibition of apoptosis may contribute to the transformation of cells or to the development of chemotherapy resistance. Mutations in apoptosis-regulatory genes (e.g., p53, PTEN, and bcl-2 and its homologues) are involved in the pathogenesis of most human cancers. Similar mutations may also be involved in the development of chemoresistance.

### CELL BIOLOGY OF APOPTOSIS

Apoptosis is a process that develops in several phases: 1) an initiation phase, which is extremely heterogeneous and during which the biochemical pathways participating in the process depend on the apoptosis-inducing agent; 2) a decision phase, which is common to different types of apoptosis, during which the cell "decides" to commit suicide; and 3) a common degradation phase, which is characterized by the activation of catabolic hydrolases (caspases and nucleases) (1-4). Although the activation of caspases (cysteine proteases cleaving at aspartic

acid [Asp] residues) and nucleases is necessary for the acquisition of the full apoptotic morphology, it appears clear that inhibition of such enzymes does not inhibit cell death induced by a number of different triggers: Bax (5,6), Bak (7), c-Myc (7), PML (8), FADD (9), glucocorticoid receptor occupancy (10,11), tumor necrosis factor (12), growth factor withdrawal (13), CXCR4 cross-linking (14), and chemotherapeutic agents, such as etoposide (10,11,15), camptothecin (16), or cisplatin (17). In the absence of caspase activation, cells manifest a retarded cytolysis without characteristics of advanced apoptosis, such as total chromatin condensation, oligonucleosomal DNA fragmentation, and formation of apoptotic bodies (5-7,10,11,18). However, before cells lyse, they do manifest a permeabilization of both mitochondrial membranes with dissipation of the inner transmembrane potential ( $\Delta\Psi_m$ ) and/or the release of apoptogenic proteins, such as cytochrome c and apoptosis-inducing factor (AIF) via the outer membrane (5,6,10,11,14,19-21). These results have invalidated the hypothesis that caspase activation is always required for apoptotic cell death to occur. Rather, cell death is intimately associated with the permeabilization of mitochondrial membranes (3).

The understanding of apoptosis has recently been facilitated by the development of cell-free systems. Instead of considering the cell as a black box, subcellular fractions (e.g., mitochondria, nuclei, and cytosols) are mixed together with the aim to reconstitute the apoptosis phenomenon by recapitulating the essential steps of the process *in vitro* (19,22-31). With the use of this approach, we have demonstrated that apoptosis of mammalian cells develops in several steps (see Fig. 1). Schematically, it appears that proapoptotic second messengers, whose nature depends on the apoptosis-inducing agent, accumulate in the cytosol during the initiation phase. These agents then induce mitochondrial membrane permeabilization, allowing cells to enter the decision phase. The apoptotic changes of mitochondria consist in a  $\Delta\Psi_m$  loss, transient swelling of the mitochondrial matrix, mechanical rupture of the outer membrane and/or its nonspecific permeabilization by giant protein-permeant pores, and release of soluble intermembrane proteins (SIMPs) through the outer membrane (5,6,10,11,19-21,32). Once the mitochondrial membrane barrier function is lost, several factors, e.g., the metabolic consequences at the bioenergetic level, the loss of redox homeostasis, and the perturbation of ion homeostasis, contribute to cell death. The activation of proteases (caspases) and nucleases by

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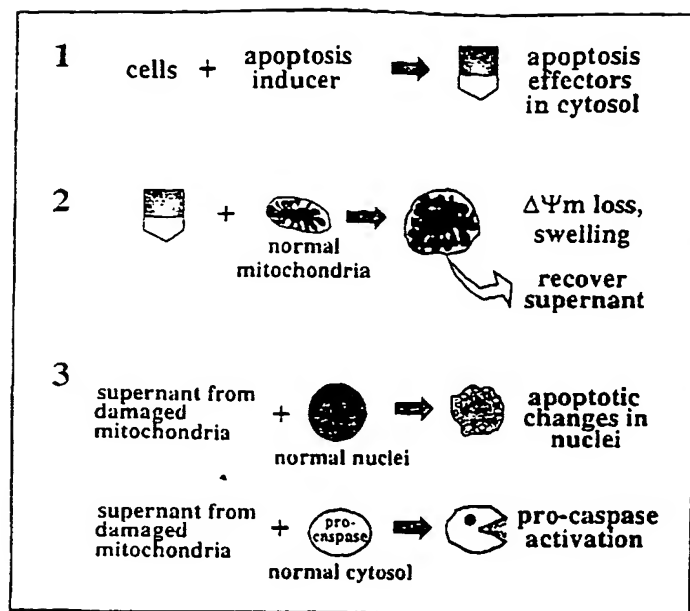


Fig. 1. Phases of the apoptotic process reconstituted in a cell-free system. Cells are treated for a short period with apoptosis inducers (e.g., anti-Fas antibody or ceramide) followed by recovery of their cytosols. This phase (1) corresponds to the premitochondrial phase and leads to the accumulation of cytosolic effector molecules. During a second, mitochondrial phase (2), these cytosols are added to purified mitochondria, isolated from untreated healthy cells, and the consequences of mitochondrial membrane permeabilization (inner transmembrane potential [ $\Delta\Psi_m$ ] loss, matrix swelling, and release of intermembrane proteins) are monitored. The supernatants of mitochondria, which contain soluble proteins normally located in the intermembrane space, can be used in a third step of the assay (3) corresponding to the postmitochondrial phase. During this step, proteins released from the mitochondrial intermembrane space provoke apoptotic changes (e.g., chromatin condensation and DNA degradation) in nuclei purified from normal cells. Alternatively, if added to cytosols from normal cells, such supernatants may favor the proteolytic activation of procaspases *in vitro*.

SIMPs is necessary for the acquisition of apoptotic morphology (4,19,22–31,33). This latter phase corresponds to the degradation step, beyond the point of no return of the apoptotic process. Different SIMPs provide a molecular link between mitochondrial membrane permeabilization and the activation of catabolic hydrolases: cytochrome *c* (a heme protein that participates in caspase activation) (34), certain procaspases (in particular, procaspases 2 and 9, which, in some cell types, are selectively enriched in mitochondria) (25,35), and AIF (19,22,24). AIF is a nuclear-encoded intermembrane flavoprotein that translocates to the nucleus where it induces the caspase-independent peripheral chromatin condensation and the degradation of DNA into 50-kilobase-pair fragments (24).

#### MOLECULAR MECHANISMS OF MITOCHONDRIAL MEMBRANE PERMEABILIZATION

The mechanism of mitochondrial membrane permeabilization is not completely understood. Some investigators prefer the hypothesis that proapoptotic members of the Bcl-2 family are inserted in the outer membrane (36) where they oligomerize and form cytochrome *c* permeant pores in an autonomous fashion, not requiring the interaction with other mitochondrial membrane proteins (37,38). However, Bax-induced membrane permeabilization is inhibited by cyclosporin A (CsA) and bongkrekic acid (BA), two inhibitors of formation of the permeability transition

pore (or "megachannel") (6,39–41), suggesting that sessile mitochondrial proteins (the targets of CsA and BA) are involved in this process. The permeability transition pore has a polypeptide structure that is formed at the contact sites between the inner and outer membranes (40,42–45). One of the key proteins of the permeability transition pore complex (PTPC) is the adenine nucleotide translocator (ANT). ANT, the target of BA, is the most abundant inner membrane protein. ANT normally functions as a specific carrier protein for the exchange of adenosine triphosphate (ATP) and adenosine diphosphate (ADP), but it can become a nonspecific pore (Fig. 2, A–C). Schematically, ANT thus has two functions—that of a vital ATP/ADP function as a carrier and that of a lethal pore (42,46–48) (Fig. 2, A). ANT interacts with another equally abundant protein of the outer membrane, the voltage-dependent anion channel (VDAC/porin), as well as a soluble protein of the mitochondrial matrix, cyclophilin D, the target of CsA (43,44). ANT and/or VDAC physically interact with Bcl-2 and Bax (41,42,49), the peripheral benzodiazepine receptor (PBR), as well as with several proteins involved in the regulation of energy metabolism (e.g., hexokinase II and creatine kinase) (40,50–52) (Fig. 2, C). It appears that the PTPC simultaneously controls the permeability of the outer membrane (pore forming protein: VDAC and/or Bax) (49) and that of the inner membrane (pore-forming protein: ANT perhaps in collaboration with Bax) (Fig. 2, B) and participates at energy metabolism (via the kinases and ANT). Because of the presence of multiple proteins, each of which influences pore opening, the PTPC may constitute a regulatory crossroad, which senses a large number of metabolic conditions: redox couples (e.g., reduced versus oxidized glutathione, nicotinamide adenine dinucleotide [ $\text{NAD}^+$ ] versus nicotinamide adenine dinucleotide phosphate [ $\text{NADPH}$ ], local ATP/ADP concentrations, different metabolites (e.g., glucose and creatine), ions ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ), the pH, and the  $\Delta\Psi_m$  (Fig. 2, C). All of these factors determine the probability of membrane permeabilization by the PTPC (53).

We have developed a protocol allowing for the reconstitution of the purified PTPC in artificial liposomes followed by the quantification of the opening and closure of PTPC (40,42,54–58) (Fig. 3). The functional and biochemical characterization of such proteoliposomes has revealed similarities between the reconstituted PTPC (in liposomes) and the natural PTPC (in mitochondria). Thus, their regulation by pro-oxidants, thiol cross-linkers, calcium, ANT ligands, and several members of the Bcl-2 family is similar. Recombinant Bcl-2 as well as Bcl-X<sub>L</sub> (both of which are antiapoptotic) have a direct inhibitory effect on the PTPC (40,42,54–58), as well as on ion channel formation by purified ANT (48) and VDAC (49). In contrast, Bax (which is proapoptotic) cooperates with ANT (42,48) or VDAC (49) to create large channels.

An interesting property of the PTPC is that the permeabilization of the inner and/or outer mitochondrial membranes compromises the bioenergetic equilibrium of the cell (e.g., it provokes the oxidation of reduced NADPH and glutathione, the depletion of ATP, and the dissipation of  $\Delta\Psi_m$ ) and affects the homeostasis of intracellular ions (e.g., by releasing  $\text{Ca}^{2+}$  from the matrix). Intriguingly, all of these changes themselves increase the probability of PTPCs opening (53). This has two important implications. First, the consequences of PTPC opening themselves favor opening of the PTPC in a self-amplification loop that coordinates the lethal response among mitochondria within the same cells. Second, this implies that the



final result of PTPC opening is a stereotyped ensemble of biochemical alterations, which does not depend on the initiating stimulus, be it a specific proapoptotic signal transduction cascade or nonspecific damage at the energy or redox levels.

Is the PTPC (and its components) the only mechanism by which mitochondrial membranes are permeabilized? The answer is not clear. Although apoptosis is almost universally accompanied by a loss of the  $\Delta\Psi_m$  and although PTPC inhibitors (CsA, BA, and/or proteins of the Bcl-2 family) frequently inhibit the mitochondrial and postmitochondrial manifestations of apoptosis, it cannot be excluded that additional mechanisms exist. Thus, proapoptotic members of the Bcl-2 family, such as Bax or Bid, may cause outer-membrane permeabilization without inducing an immediate  $\Delta\Psi_m$  dissipation (37,38). Apoptosis without a complete  $\Delta\Psi_m$  loss has also been reported to occur in some cell lines, such as HL60 (21). Because Bax can kill yeast cells lacking VDAC expression, at least in some experimental settings (59), it is possible that Bax (and other proapoptotic members of the Bcl-2 family?) may act in an autonomous fashion, i.e., by forming giant channels and/or acting on mitochondrial structures other than the PTPC.

### NEOPLASIA AND CHEMOTHERAPY: ROLE OF MITOCHONDRIA

The composition of the PTPC (Fig. 2, C) is different in normal and in malignant cells. It is tempting to relate this difference to the mechanisms of tumorigenesis, in particular to the acquisition of apoptosis resistance and cancer-specific metabolic alterations.

The gene coding for ANT2 (one of the three ANT isoenzymes), whose expression is normally repressed in quiescent cells, is transcribed in dedifferentiated, proliferating tumor cells (60,61). Three other putative PTPC components (Fig. 2, C), i.e., PBR, the PBR-associated protein Prax-1, and mitochondrial creatine kinase, are also overexpressed in some tumors (52,62–65). Intriguingly, mitochondrial creatine kinase can confer apoptosis resistance (and inhibition of the PTPC) in the presence of creatine (52,64). Moreover, overexpression of PBR confers a relative resistance to oxidative stress (66). More important, the expression of functional Bax is frequently reduced in cancer cells, either at the transcriptional level or because of loss-of-function mutations (67–71), and Bcl-2 or its antiapoptotic homologues are overexpressed in a large percentage of neoplasias (72–75). This strengthens the hypothesis that the composition and/or the control of the PTPC can be altered in tumors.

Cancer cells withstand an adverse microenvironment (hypoxia, acidosis, hypoglycemia, and shortage of growth factors) by virtue of metabolic adaptation. Solid tumors are characterized by a resistance to hypoxia coupled to an increased anaerobic glycolysis that is not influenced by the oxygen concentration (Warburg effect) (76–78). The Warburg effect is still not fully understood. In this context, it may be intriguing that the hypoxia-inducible transcription factor, in conjunction with mutant p53 protein, accounts for the overexpression of the glycolytic enzyme hexokinase II (79), which is associated with VDAC in normal brain and in a variety of tumors (but not in other normal tissues) (80). For instance, mitochondria from hepatoma or hepatocellular carcinoma do bind hexokinase II, but normal liver cell mitochondria do not (78). Whether this or additional alterations in the composition of the PTPC may explain the Warburg effect remains an open question.

Chemotherapy aims at the specific eradication of cancer cells, mostly through the induction of apoptosis. What is the practical implication of the mitochondrial control of apoptosis? As mentioned above, mitochondrial membrane permeabilization is a near-to-general feature of apoptosis (2,4,81). The mere detection of such mitochondrial alterations thus does not distinguish between two fundamentally different options: the direct action of a chemotherapeutic drug on mitochondria or the indirect perturbation of mitochondrial function through the activation of proapoptotic signal transduction pathways and/or damage of extra-mitochondrial structures. To distinguish between these possibilities, experiments have to be performed in which the effects of the anticancer drugs on isolated mitochondria (step 2 in Fig. 1) or on purified PTPC (Fig. 3) are evaluated. One particularly interesting possibility consists in combining different cellular components (i.e., nuclei, cytosols, mitochondria, etc.) in a cell-free system to study the minimum requirement for the activation of caspases or the induction of nuclear apoptosis by anticancer agents. Several conventional anticancer agents, such as etoposide, doxorubicin, or cisplatin, have no direct effect on mitochondria (82). Instead, they activate signal transduction pathways that are also involved in physiologic apoptosis. In the following sections, we will discuss which of the endogenous and xenobiotic agents have a direct effect on mitochondria.

### PROAPOPTOTIC SIGNAL-TRANSDUCING MOLECULES ACTING ON MITOCHONDRIA

Conventional anticancer agents, such as etoposide, doxorubicin, cisplatin, or paclitaxel (Taxol), trigger apoptosis by inducing p53 expression; by inducing the ceramide/GD3 pathway; by inducing the CD95/CD95L ligand system, affecting Bcl-2-like proteins; and/or by compromising the redox or energy balance. Thus, these agents cause mitochondrial permeabilization in an indirect fashion by eliciting perturbations of intermediate metabolism or by increasing the concentration of proapoptotic second messengers. Which among these endogenous effectors do exert a direct effect on isolated mitochondria or on the reconstituted PTPC?

**Redox metabolism.** An enhanced generation of reactive oxygen species is not always the result of cellular damage; it also can result from overexpression of the proapoptotic antioncogene p53 (83) or from treatment of cells with the second messenger ceramide (84). Changes in cellular redox potentials due to an enhanced generation of reactive oxygen species (or a decrease in their detoxification), depletion of nonoxidized glutathione, or depletion of NADPH suffice to induce or to facilitate PTPC opening (53,85,86). Peroxynitrite (which is formed by the reaction of nitric oxide with superoxide anion) is also a potent PTPC trigger (87,88) (Fig. 4). The mitochondrial megachannel possesses several redox-sensitive sites, one that is modulated by NADPH and another that is in equilibrium with mitochondrial matrix glutathione (89). This latter site is likely to be Cys56 of the ANT, based on the observation that oxidation of this thiol (which is exposed to the matrix) suffices to convert ANT into a large nonspecific pore (90).

**Energy metabolism.** ADP and ATP are the physiologic ligands of the adenine nucleotide translocator and function as endogenous inhibitors of the PTPC (53,85,86,91) and/or pore formation by ANT induced by atractyloside, reactive oxygen species, or thiol cross-linkers (48,90). Their depletion, therefore, might facilitate PTPC opening. Matrix alkalinization and/or

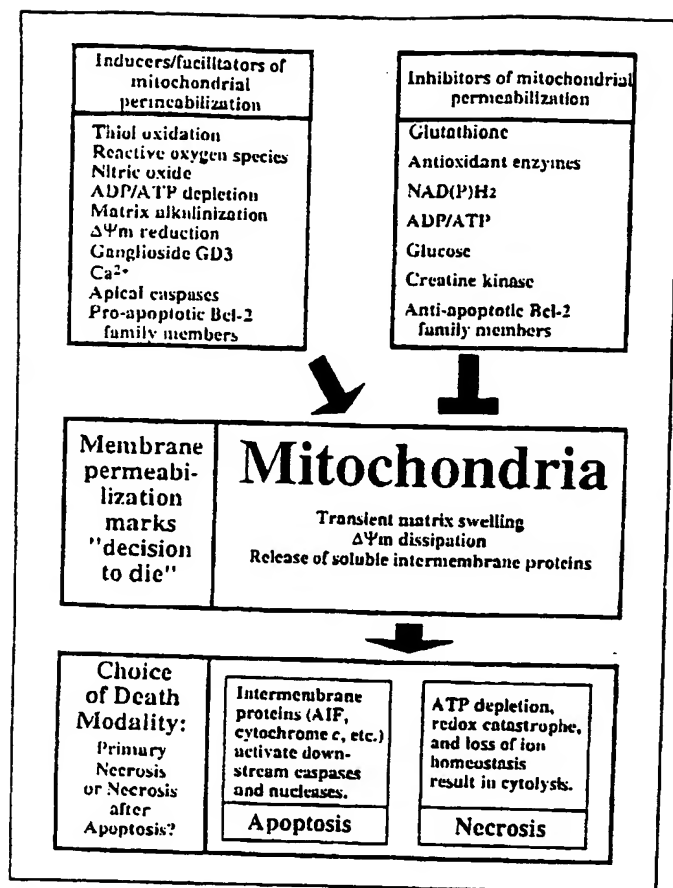


Fig. 4. Phases of the apoptotic process. During the premitochondrial phase (initiation phase), the accumulation of apoptosis inducers (or the depletion of inhibitors) results in mitochondrial membrane permeabilization. This phase is heterogeneous, and the nature of the second messenger depends on the lethal trigger that initiates the process. Mitochondrial membrane permeabilization due to opening of the permeability transition pore complex or perhaps due to alternative mechanisms constitutes the decisive event of cell death, provoking the loss of the mitochondrial transmembrane potential ( $\Delta\Psi_m$ ) and/or the permeabilization of the outer membrane. Mitochondrial permeabilization is the first event of the common apoptotic pathway and marks the point of no return of the process (mitochondrial phase or decision phase). Soluble intermembrane proteins are liberated from mitochondria and activate caspases and nucleases, provided that there is sufficient energy to maintain the integrity of the cell during the activation of these hydrolases. The acquisition of the apoptotic morphology occurs during the degradation phase (postmitochondrial phase). According to this scheme, the biochemical event that seals the cell's fate is mitochondrial membrane permeabilization, irrespective of the death modality (apoptosis or necrosis). ADP = adenosine diphosphate; ATP = adenosine triphosphate; NAD(P)H = reduced nicotinamide adenine dinucleotide phosphate; NAD = nicotinamide adenine dinucleotide; and AIF = apoptosis-inducing factor.

$\Delta\Psi_m$  reduction also trigger PTPC opening (53,85,86). Thus, uncoupling or inhibition of the respiratory chain (which leads to a decrease in  $\Delta\Psi_m$ ) may be expected to favor mitochondrial membrane permeabilization.

**Lipid messengers.** Ceramide is generated in cells exposed to several apoptosis-inducing stimuli, including signaling via the Fas/Apo-1/CD95 receptor or the tumor necrosis factor receptor, nonspecific stress, or cytotoxic drugs (92). When added to cells, ceramide induces mitochondrial membrane permeabilization (23,93,94) but does not induce PTPC opening in isolated mitochondria (23). To induce apoptosis, ceramide must be converted

into ganglioside GD3 in the Golgi apparatus (94). GD3 translocates to mitochondria and causes PTPC opening in a direct fashion, since this has been demonstrated both in isolated mitochondria and in intact cells (95). In addition to GD3, fatty acid palmitate (which interacts with ANT) (96) and lipid oxidation product 4-hydroxyhexenal (97) can induce PTPC opening when they are added to purified mitochondria.

**Cytosolic calcium.**  $\text{Ca}^{2+}$  ions are among the most effective triggers of the PTPC. At supraphysiologic doses ( $>10 \mu\text{M}$   $\text{Ca}^{2+}$  suffices to induce permeability transition (PT); at low doses, it facilitates the induction of PT by other stimuli (53). Increases in free  $\text{Ca}^{2+}$  concentrations are also important cofactors of apoptotic cell death. It is unknown as to what extent  $\text{Ca}^{2+}$  triggers cell death via direct mitochondrial effects. However, it appears that Bcl-2 overexpression enhances the tolerance of mitochondria to  $\text{Ca}^{2+}$  (98).

**Proapoptotic members of the Bcl-2 family.** On induction of apoptosis, several proapoptotic Bcl-2 family members can translocate from the cytosol (Bax, Bid, and Bad) or from microtubules to mitochondria, where they incorporate into the outer membrane and may undergo a conformational change (36,99,100). The mechanism of Bax translocation is unclear (99), but Bid translocation may involve its cleavage by caspase 8 (101), and Bad translocation may involve its dephosphorylation, causing its release from cytosolic 14-3-3 protein (102). Bax and Bak cause mitochondrial membrane permeabilization in a way that is inhibited by the PTPC inhibitors CsA and I (39,41,42), although this conclusion has been contested (3). Proapoptotic signaling may also lead to the inactivation of antiapoptotic members of the Bcl-2 family. Inactivation of Bcl-2 achieved by chemotherapeutic agents (such as paclitaxel), which act on microtubule assembly and cause its hyperphosphorylation (103) and, simultaneously, favor opening of the PT pore (104). In addition, Bcl-2 can be cleaved by caspase 3 in a reaction that yields a proapoptotic product (105).

**Caspases.** Ligation of some receptors can lead to a rapid proteolytic activation of caspases within seconds or minutes. This was first documented for the Fas/Apo-1/CD95 receptor, which, on interaction with the Fas/Apo-1/CD95 ligand, recruits caspase 8 into the receptor complex and causes its activation. Caspases can act on members of the Bcl-2 family (e.g., caspase 8 cleaves Bid, caspase 1 cleaves Bcl-XL, and caspase 3 cleaves Bcl-2; see above), thereby activating proapoptotic members of the Bcl-2 family (Bid) or inactivating antiapoptotic members (Bcl-2 and Bcl-XL). In the Fas/Apo-1/CD95-triggered pathway, mitochondrial membrane permeabilization (mediated by Bid or ceramide/GD3) may be a prerequisite of cell death ("type 1 cells") but may be dispensable when caspase 8 activates other caspases in a direct fashion ("type 2 cells") (106,107).

## CHEMOTHERAPEUTIC AGENTS ACTING DIRECTLY ON MITOCHONDRIA

A large number of toxins inhibit the respiratory function of mitochondria and/or stimulate futile redox cycles, thereby inducing cell death. Two strategies may be employed to target toxic agents to mitochondria. The first strategy uses agents that interact specifically with mitochondrial proteins; the second strategy exploits the fact that lipophilic cations accumulate in the mitochondria matrix, driven by the electrochemical gradient. According to the Nernst equation, every 61.5-mV increase in

$\Delta\Psi_m$  (usually 120–170 mV, negative inside) leads to a 10-fold increase in cation concentration in the mitochondrial matrix. Therefore, the concentration of such cations is two to three logs higher in the matrix than in the cytosol. Several types of cancer cells have been described to accumulate such agents, e.g., rhodamine 123, to a higher level than normal cells (108). Attempts have been made to use cationic lipophilic toxins as antitumor agents. MKT-077, a cationic rhodacyanine dye, is selectively toxic to carcinoma cells *in vitro* and *in vivo* (109), perhaps owing to the higher  $\Delta\Psi_m$  in carcinoma cells versus normal cells (108). Whether the putative elevation of  $\Delta\Psi_m$  in cancer cell actually explains the MKT-077 effects, however, remains unclear. On theoretic grounds, selection for tumor cells bearing a slightly reduced  $\Delta\Psi_m$  would suffice to confer drug resistance. Two mechanisms accounting for the mitochondrial toxicity of MKT-077 have been proposed, namely, a selective MKT-077-driven depletion of mitochondrial DNA in carcinoma cells but not in

normal epithelial cells and inhibition of mitochondrial respiration with a decrease in the activities of succinate–cytochrome c reductase and cytochrome oxidase. All of these effects are enhanced by photoactivation (110). Because of its selectivity toward tumor cells, MKT-077 is currently being evaluated in phase I clinical trials (109). Another cationic lipophilic dye, chloromethyl-X-rosamine, also acts as a photosensitizer (111). In addition to these dyes, a number of experimental chemotherapeutic agents have been reported to permeabilize mitochondrial membranes (Table 1).

Lonidamine, i.e., 1-[(2,4-dichlorophenyl)methyl]-1H-indazole-3-carboxylic acid (LND), is an antitumoral drug derived from indazole-3-carboxylic acid. LND enhances the apoptotic response to cisplatin, cyclophosphamide, doxorubicin, paclitaxel, melphalan, and  $\gamma$ -irradiation both *in vitro* and *in vivo*. LND has been used in combination chemotherapy in phase II and III trials in patients with metastatic breast cancer (112,113) and

Table 1. Chemotherapeutic drugs acting on mitochondria\*

Name	Class	<i>In vitro</i> and <i>in vivo</i> studies	Phase II or III trials	Inhibition by Bcl-2	Effects on mitochondria confirmed in	Other apoptotic effects
Arsenite	Arsenic trioxide	Induction of apoptosis in APL, NHL, B-CLL, plasma and ovarian carcinoma cells	APL	Yes	Intact cell† Mitochondria‡ PTPC§	Decrease of Bcl-2 Suppression of the apoptosis-inhibitory Ras/MAP kinase cascade Activation of PML or PML-RAR $\alpha$
Betulinic acid	Pentacyclic triterpene	Induction of apoptosis of neuroectodermal, melanoma, and glioma cells		Yes	Intact cell† Mitochondria‡	Increase of Bcl-2 and Bax
PK11195, RO5-4864, diazepam	Ligands of the PBR	Human B- and T-cell lines, osteosarcoma, neuroblastoma, and glioma cells		Yes	Intact cell† Mitochondria‡	
Lonidamine	Indazole-3-carboxylic acid	Enhancement of cisplatin, doxorubicin, melphalan, cyclophosphamide, paclitaxel, irradiation, diazepam, and saporin 6	Breast, ovarian, and non-small-cell lung carcinomas	Yes	Intact cell† Mitochondria‡ PTPC§	Acidosis (lactate) Loss of energy (ATP and aerobic glycolysis) Increase cytosolic Ca <sup>2+</sup> Interaction with hexokinase
MKT-077	Rhodacyanine dye	Induction of apoptosis of colon, breast, pancreatic, gastric, bladder, and prostatic carcinomas and melanoma			Mitochondria† Inhibition of respiration in cells‡	
CD437	Synthetic retinoid	Induction of apoptosis in APL, human lung, breast, cervical, and ovarian carcinoma cells; melanoma cells; prostate cancer cells; and neuroblastoma cells		No	Intact cell† Mitochondria‡	Caspase 3 and caspase 7 activation Degradation of PML-RAR $\alpha$ protein Increase of p53, p21, and Bax Activation of AP-1 complex Decrease of Bcl-X <sub>L</sub> S-phase arrest
(KLAKKLAK) <sub>2</sub> Vpr	$\alpha$ -Helical peptides with positive charges	Broad cytotoxic effects: (KLAKKLAK) <sub>2</sub> may be targeted to angiogenic epithelial cells <i>in vitro</i> and <i>in vivo</i>		Yes (for Vpr)	Intact cell† Mitochondria‡ PTPC§	Vpr blocks cell cycle at G <sub>2</sub>

\*APL = acute promyelocytic leukemia; NHL = non-Hodgkin's lymphoma; B-CLL = B-chronic lymphoid leukemia; PTPC = permeability transition pore complex; PML = promyelocytic leukemia; RAR $\gamma$  = retinoic acid receptor  $\gamma$ ; PBR = peripheral benzodiazepine receptor; ATP = adenosine triphosphate; APL = acute promyelocytic leukemia; Vpr = viral protein R.  $\Delta\Psi_m$  = mitochondrial inner membrane potential; and (KLAKKLAK)<sub>2</sub> KLAKKLAKKLAKKLAK (peptide sequence in which K = lysine, L = alanine, and L = leucine).

†Cellular apoptosis: drop of the  $\Delta\Psi_m$ , generation of reactive oxygen species; DNA fragmentation, loss of cell viability, and liberation of mitochondrial factors (cytochrome c and apoptosis-inducing factor).

‡Effects on isolated mitochondria: loss of the  $\Delta\Psi_m$ , large-amplitude swelling, and release of soluble intermembrane proteins.

§Effects on the purified PTPC reconstituted into liposomes, resulting in membrane permeabilization.



in those with inoperable non-small-cell lung cancer (NSCLC) (114), demonstrating an improvement in the overall response rate for both tumors and of the median time to progression and median survival time for NSCLC. Recently, it has been found that LND exerts a direct effect on the mitochondrial permeability transition pore (56). In different cell lines, LND induces a drop in the mitochondrial transmembrane potential ( $\Delta\Psi_m$ ) preceding generation of reactive oxygen species, DNA fragmentation, and cell viability. In cell-free systems of nuclear apoptosis, LND has no direct effect and requires the addition of mitochondria to cause chromatin condensation and DNA fragmentation. When added to isolated mitochondria, LND causes the dissipation of the  $\Delta\Psi_m$  and the release of apoptogenic factors, such as cytochrome c. All of these effects are reduced by the PTPC inhibitor CsA. When added to liposomes containing the PTPC, LND permeabilizes membranes, but no such effects are found on control liposomes lacking the PTPC. All apoptotic effects of LND on intact cells, isolated mitochondria, or purified PTPC are inhibited by recombinant Bcl-2 protein (56,115). Taken together, these data support the hypothesis that LND induces apoptosis via a direct action on the PTPC and that Bcl-2 blocks the LND effects via its PTPC-inhibitory function.

Arsenite (the trivalent inorganic salt formed by arsenic trioxide) recently has become a therapeutic agent of choice for the treatment of acute promyelocytic leukemia (APL) (116). Moreover, human T-cell leukemia/lymphotropic virus type I (HTLV-I)-infected cells, myeloma cells, and transformed lymphocytes are extremely sensitive to arsenic (117,118). Cell-free systems of apoptosis have revealed that arsenite requires mitochondria to induce nuclear apoptosis *in vitro* (58). Moreover, arsenite acts on isolated mitochondria to induce PTPC opening (89). Since arsenite toxicity is modulated by the reduced glutathione content (119), it may be speculated that it acts as a thiol-oxidizing agent (89). However, arsenite does not cause oxidation of Cys56 of ANT as other thiol-reactive agents do (90). Arsenite acts on the purified PTPC reconstituted into liposomes *in vitro* (58). In such a system, recombinant Bcl-2 prevents PTPC opening. This parallels the observation that transfection-mediated overexpression of Bcl-2 protects cells against the proapoptotic effect of arsenite (58).

CD437 (6[3-adamantyl-4-hydroxyphenyl]-2-naphthalene carboxylic acid) is a new synthetic retinoid acid receptor  $\gamma$  (RAR $\gamma$ ) agonist inducing apoptosis of human breast, lung, cervical, and ovarian carcinomas, melanoma, prostate cancer cells, neuroblastoma, and APL. Several mechanisms of induction of the cell-death process have been reported: activation of AP-1 complex (120); increase of p53, p21, and Bax (121); decrease of Bcl-X<sub>L</sub> (122); cell-cycle arrest; and activation of caspase 3 and caspase 7. Although it was tacitly assumed that CD437 acts via RAR $\gamma$ , CD437 can also kill RAR $\gamma$ -negative cell lines (123) and cytoplasts (i.e., cells without a nucleus) (124). Thus, CD437-dependent apoptosis does not require activation of RAR $\gamma$  or that of any other nuclear RAR. Moreover, in intact cells CD437-dependent caspase activation is preceded by the release of cytochrome c from mitochondria, and this release is not affected by the caspase inhibitor Z-VAD-fmk (124). CD437 also causes membrane permeabilization when added to purified mitochondria, and this effect is prevented by the PTPC inhibitors CsA and BA. Since CD437-mediated cell killing is suppressed by CsA and BA (124), it appears plausible that CD437 exerts its cytotoxic effects via the PTPC.

Betulinic acid, a pentacyclic triterpene, is a novel experimental anticancer drug. It possesses an antitumoral activity *in vitro* and *in vivo* in melanoma, neuroectodermal tumors, and glioma cell lines. Fulda et al. (82) have shown that betulinic acid induces apoptosis via direct mitochondrial alterations. All of these effects have been observed in intact cells and in cell-free systems. When added to isolated mitochondria, betulinic acid directly induces loss of  $\Delta\Psi_m$  in a way that is not affected by the caspase inhibitor Z-VAD-fmk and yet is inhibited by BA. In a cell-free system comprising mitochondria, cytosols, and purified nuclei, mitochondria undergoing betulinic acid-induced permeability transition mediate cytosolic caspase activation (caspase 8 and caspase 3) and nuclear fragmentation via the liberation of soluble factors, such as cytochrome c or AIF (125). Bcl-2 and Bcl-X<sub>L</sub> block all mitochondrial and cellular manifestations of apoptosis induced by betulinic acid, as does BA, an inhibitor of the PTPC (125).

## DRUG DESIGN: PROAPOPTOTIC PEPTIDES TARGETED TO MITOCHONDRIA

Gene therapy can employ Bax-delivering vectors, thereby indirectly targeting mitochondria to induce apoptosis (126,127). In contrast to such proteins, certain peptides readily penetrate the plasma membrane and thus can be used as true pharmacologic agents. Mastoparan, a peptide isolated from wasp venom, is the first peptide known to induce mitochondrial membrane permeabilization via a CsA-inhibitable mechanism (128) and to induce apoptosis via a mitochondrial effect when added to intact cells (129). This peptide has an  $\alpha$ -helical structure and possesses some positive charges that are distributed on one side of the helix. A similar peptide (KLAKLAKKLAKLAK or (KLAKLAK)<sub>2</sub> (K = lysine, L = alanine, and A = leucine) has been found recently to disrupt mitochondrial membranes (130) when it is added to purified mitochondria, although the mechanisms of this effect have not been elucidated. The proapoptotic 96 amino acid protein viral protein R (Vpr) from human immunodeficiency virus-1 contains a comparable structural motif (aa 71–82), i.e., an  $\alpha$ -helix with several cationic charges that concentrate on the same side of the helix (131). We recently have shown that Vpr, as well as Vpr derivatives containing this "mitochondriotoxic" domain cause a rapid CsA- and BA-inhibited dissipation of the  $\Delta\Psi_m$  as well as the mitochondrial release of apoptogenic proteins, such as cytochrome c or AIF (132). The same structural motifs relevant for cell killing appear to be responsible for the mitochondriotoxic effects of Vpr. Vpr favors the permeabilization of artificial membranes containing the purified PTPC or defined PTPC components such as the ANT combined with Bax, but this effect is prevented by the addition of recombinant Bcl-2. According to surface plasmon resonance studies, the Vpr C-terminus binds purified ANT with a high affinity in the nanomolar range (132). In addition, a biotinylated Vpr-derived peptide (Vpr52–96) may be employed as bait to specifically purify a mitochondrial molecular complex containing ANT and the VDAC. Yeast strains lacking ANT or VDAC are less susceptible to Vpr-induced killing than are control cells. Thus, Vpr induces apoptosis via a direct effect on the mitochondrial PTPC (132). In analogy to Vpr, the p13 (II) protein derived from the X-II open reading frame of HTLV-1 is targeted to mitochondria and can cause a dissipation of the  $\Delta\Psi_m$  and mitochondrial swelling. Mitochondrial targeting of this protein has been mapped to a decapeptide sequence that contains several

Arg residues that are asymmetrically distributed in the  $\alpha$ -helix. However, Arg→Ala substitutions within the mitochondriotoxic domain of p13 (II) did not abolish the mitochondrial targeting of p13 (133).

The data discussed above suggest that lethal peptides may be targeted to mitochondria and more specifically, at least in the case of Vpr, to the PTPC. Ellerby et al. (130) recently have fused the mitochondriotoxic (KLAKLAK)<sub>2</sub> motif to a targeting peptide that interacts with endothelial cells. Such a fusion peptide is internalized and induces mitochondrial membrane permeabilization in angiogenic endothelial cells and kills MDA-MD-435 breast cancer xenografts transplanted into nude mice. Similarly, a recombinant chimeric protein containing interleukin 2 (IL-2) protein fused to Bax selectively binds to and kills IL-2 receptor-bearing cells *in vitro* (134). Thus, specific cytotoxic agents that target surface receptors, translocate into the cytoplasm, and induce apoptosis via mitochondrial membrane permeabilization might be useful in treating cancer.

## TWO STRATEGIES TO OVERCOME BCL-2-MEDIATED INHIBITION OF APOPTOSIS AT THE MITOCHONDRIAL LEVEL

Bcl-2 and similar antiapoptotic proteins confer protection against most of the endogenous or xenobiotic effectors acting on mitochondria (see above). One strategy to overcome Bcl-2-mediated cytoprotection consists in the use of thiol cross-linkers, including diazenedicarboxylic acid bis 5*N,N*-dimethylamide (diamide), dithiodipyridine (DTDP), or bis-maleimido-hexane (BMH) (54,90). Such agents act on the ANT (90). ANT alone reconstituted into artificial lipid bilayers suffices to confer a membrane permeabilization response to thiol cross-linking agents. Diamide, DTDP, and BMH, but not tert-butylhydroperoxide or arsenite, cause the oxidation of a critical cysteine residue (Cys 56) of ANT. Thiol modification within ANT is observed in intact cells, isolated mitochondria, and purified ANT. Recombinant Bcl-2 fails to prevent thiol modification of ANT. Concomitantly, a series of different thiol cross-linking agents (diamide, DTDP, BMH, and phenylarsine oxide), but not tert-butylhydroperoxide or arsenite, induce mitochondrial membrane permeabilization and cell death irrespective of the expression level of Bcl-2. These results indicate that thiol cross-linkers cause a covalent modification of ANT which, beyond any control by Bcl-2, leads to mitochondrial membrane permeabilization and cell death (90). Because the Cys56 of ANT is located at the matrix site of the inner mitochondrial membrane, it may be possible to design positively charged thiol cross-linkers that can specifically accumulate at this site of the inner membrane (negative charges inside) following the Nemst equation. On theoretic grounds, such positively charged yet lipophilic thiol cross-linkers would have a higher cytotoxic potential than noncharged cross-linkers. Whether such agents may be used in chemotherapy, however, remains an open question.

A second strategy to overcome Bcl-2-mediated cytoprotection consists in the use of ligands of the PBR (135,136), an outer mitochondrial membrane protein that physically interacts with VDAC and ANT (50). Ligands of the mitochondrial benzodiazepine receptor, such as PK11195, can overcome the apoptosis resistance of Bcl-2-overexpressing cells in response to diverse stimuli, including glucocorticoids (136), the topoisomerase inhibitor etoposide (136), LND (56), or arsenite (58). PK11195 also abolishes the resistance of Bcl-2-overexpressing mitochondria

to the induction of PTPC opening by the ANT ligand atractyloside *in vitro* (136). Protoporphyrin IX, a PBR ligand, can also override Bcl-2-mediated cytoprotection (135). Verteporfin, a porphyrin-derived photosensitizer, similarly causes mitochondrial membrane permeabilization irrespective of Bcl-2 or Bcl-XL overexpression (137). Diazepam, also a ligand of the mitochondrial peripheral benzodiazepine receptor, and LND have synergistic effects in the treatment of nude mice bearing human glioblastoma (138).

## PERSPECTIVES

A recurrent problem with conventional chemotherapeutic agents is that they exploit endogenous apoptosis-induction pathways that may be compromised by alterations, such as mutations of p53, increased antioxidant activity, blockade of the CD95/CD95L pathway, overexpression of Bcl-2-like proteins, etc. One possible strategy to enforce cell death is to trigger downstream events of the common apoptotic pathway. Thus, adenovirus-mediated transfer of caspases has been proposed as one strategy to induce cell death beyond any regulation (139). An alternative strategy is to use mitochondriotoxic agents that induce cell death irrespective of the upstream control mechanisms and irrespective of the status of caspases and endogenous caspase inhibitors. As an example, LND, arsenite, or CD437 induce cell death independently of the p53 status (121,140,141) via a pathway that is not affected by caspase inhibitors (56,58,124). Similarly, betulinic acid and Vpr trigger CD95 (Apo-1/Fas)- and p53-independent apoptosis (142,143), and both permeabilize mitochondrial membranes in a caspase-independent fashion (125,132). As a result, this type of agent may prove to be highly useful in killing normally resistant cells. Moreover, the future of tumor therapy may profit from the design of agents that overcome the Bcl-2-mediated stabilization of mitochondrial membranes as well as from targeting amphipathic peptides or peptidomimetics to defined cellular populations.

On theoretic grounds, selective eradication of transformed cells by use of mitochondrion-specific agents should be effective. One strategy is to target a toxic agent to selected cell types on the basis of the specific expression of surface receptors. Another, yet to be developed, strategy would aim at exploiting differences in the composition or regulation of the PTPC between normal and tumor cells. Future research will tell to which extent cell targeting (by use of retroviral or adenoviral vectors, use of integrin-specific domains, etc.) and/or targeting of tumor-specific alterations in the PTPC will prove to be useful in cancer therapy.

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## NOTES

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